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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,214,841**, on October 31, 1997, by **LISA McKERRACHER**, for "Rho Antagonists and their Use to Block Inhibition of Neurite Outgrowth".

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1

RHO FAMILY ANTAGONISTS AND THEIR USE TO
BLOCK INHIBITION OF NEURITE OUTGROWTH

FIELD OF INVENTION

This invention relates to the regulation of growth of neurons in the Central Nervous System.

BACKGROUND

Following trauma in the adult central nervous system (CNS) of mammals, injured neurons do not regenerate their transected axons. An important barrier to regeneration is the axon growth inhibitory activity that is present in CNS myelin and that is also associated with the plasma membrane of oligodendrocytes, the cells that synthesize myelin in the CNS (see Schwab, *et al.*, Ann. Rev. Neurosci., 16, 565-595, 1993 for review). The growth inhibitory properties of CNS myelin have been demonstrated in a number of different laboratories by a wide variety of techniques, including plating neurons on myelin substrates or cryostat sections of white matter, and observations of axon contact with mature oligodendrocytes (Schwab *et al.*, 1993). Therefore, it is well documented that adult neurons cannot extend neurites over CNS myelin *in vitro*.

It has also been well documented that removing myelin *in vivo* improves the success of regenerative growth over the native terrain of the CNS. Regeneration occurs after irradiation of newborn rats, a procedure that kills oligodendrocytes and prevents the appearance of myelin proteins (Savio and Schwab, Neurobiology, 87, 4130-4133, 1990). After such a procedure in rats and combined with a corticospinal tract lesion, some corticospinal axons regrow long distances beyond the lesions. Also, in a chick model of spinal cord repair, the onset of myelination correlates with a loss of its regenerative ability of cut axons (Keirstead, *et al.*, Proc. Nat. Acad. Sci. (USA), 89, 11664-11668, 1992). The removal of myelin with anti-galactocerebroside and complement in the embryonic chick spinal cord extends the permissive period for axonal regeneration. These experiments demonstrate a good correlation between myelination and the failure of axons to regenerate in the CNS.

2

Myelin inhibits axon growth because it contains at least several different growth inhibitory proteins. It has been well documented by us and by others that myelin-associated glycoprotein (MAG) has potent growth inhibitory activity, both *in vitro* and *in vivo* (McKerracher et al. 1994; Mukhopadhyay et al. 1994; Li et al. 1996; Schafer et al. 1996). A high molecular weight inhibitory activity has been characterized by Schwab and collaborators, and neutralization of this activity with the IN-1 antibody allows some axons to regenerate in white matter (Schwab et al. 1993; Bregman et al. 1995). We also have evidence that there is an additional growth inhibitory protein in myelin (Xiao et al. 1997). Clearly, there are multiple inhibitory proteins that stop axon regeneration in mammalian CNS myelin.

While axons damaged in the CNS *in vivo* do not typically regrow, there have been some reports of long distance axon extension in adult white matter. Such growth has been observed following transplantation of grafted neural tissue (Wictorin et al. 1990; Davies et al. 1994; Isacson and Deacon, 1996), suggesting that embryonic neurons primed for rapid extension of axons may be less susceptible to growth inhibition. Some embryonic neurons are not susceptible to MAG (Mukhopadhyay et al. 1994), but most embryonic neurons are inhibited by the other myelin inhibitors (Schwab et al. 1993). Therefore, in the cases when axons are able to extend on myelin, signaling through intracellular pathways may play an important role in stimulating, or blocking the inhibition of axon growth. For example, it is known that laminin is able to stimulate rapid neurite growth (Kuhn et al. 1995), and we have documented that when laminin is present in sufficient concentration, neurites can extend directly on myelin substrates. These findings suggest the possibility that the stimulation of the integrins, the receptors for laminin, is sufficient to allow axon growth on myelin. Similarly, it has been documented that when the adhesion molecule L1 is expressed ectopically on astrocytes, it can partially overcome their non-permissive substrate properties (Mohajeri et al. 1996). Therefore, neurons can, under *appropriate conditions*, grow axons on inhibitory substrates, suggesting that the balance of positive to negative growth cues is a critical determinant for the success or failure of axon regrowth after injury.

Growth inhibitory proteins typically cause growth cone collapse, a process that causes dramatic rearrangements to the growth cone cytoskeleton (Bandtlow et al. 1993; Fan et al. 1993; Li et al.

1996). One family of proteins that has been implicated in receptor-mediated signaling to the cytoskeleton is the small GTPases of the Rho family (Hall, 1996). In non-neuronal cells it has been clearly documented that mutations in Rho family members that include Rho, Rac and cdc42, affect adhesion, actin polymerization, and the formation of lamellipodia and filopodia, which are all process important to motility (Nobes and Hall, 1995). There is now good evidence that members of the Rho family regulate axon outgrowth in development. Mutations in Rho-related family members block the extension of axons in Drosophila (Luo et al. 1994) and disrupt axonal pathfinding in C. elegans (Zipkin et al. 1997). More recently it has been shown that the guidance molecule collapsin acts through a Rac-dependent mechanism (Jin and Strittmatter, 1997). In transgenic mice that express constitutively active Rac in Purkinje cells, there are alterations in the development of axon terminals and dendritic arborizations (Luo et al. 1996). Consistent with the observations *in vivo*, it was found that dominant negative Rac expressed in PC12 cells disrupts neurite outgrowth in response to NGF (Hutchens et al. 1997). Also, treatment of PC12 cells with lysophosphatidic acid, a mitogenic phospholipid, causes neurite retraction that is mediated by Rho (Tigyi et al. 1996). Therefore, different members of the Rho family can exert distinct effects on neurite growth, and in PC12 cells the activation of Rho is correlated with growth cone collapse. In non-neuronal cells, Rho participates in integrin-dependent signalling (Laudanna et al. 1996; Udagawa and McIntyre, 1996). The possibility that Rho might play a role within the myelin-derived growth inhibitory system has been studied (Jin and Strittmatter (1997) J. Neurosci. 17:6256-6263). It was concluded, however, that the inhibitory effects of myelin are not mediated by Rho family members.

A need remains for a means of inactivating the multiple inhibitory proteins present in myelin that prevent axonal regrowth after injury in the CNS.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

4

SUMMARY OF THE INVENTION

The present invention relates to antagonists and inhibitors to members of the Rho family of proteins, antibodies directed against the components of this system and diagnostic, therapeutic, and research uses for each of these aspects. In particular, members of the Rho family of proteins serve as a therapeutic target to foster regrowth of injured or degenerating axons in the CNS.

In accordance with the present invention, a preferred embodiment relates to antagonists and inhibitors of members of the Rho family of proteins and their use as a means of blocking a common signaling pathway used by the diverse growth inhibitory molecules. The antagonists and inhibitors may be either peptides or small molecules.

In yet a further aspect of the present invention, Rho family members protein can be used to design small molecules that antagonize and inhibit Rho family proteins, to block inhibition of neurite outgrowth. In another aspect of the present invention Rho family members can be used to design antagonist agents that suppress the myelin growth inhibitory system. These antagonist agents can be used to promote axon regrowth and recovery from trauma or neurodegenerative disease.

In a further aspect of the present invention, inhibitors of the Rho family of proteins can be used to block inhibition of neurite outgrowth and to suppress the myelin growth inhibitory system. Such inhibitors could block exchange of the GTP/GDP cycle of Rho activation/inactivation.

A further embodiment involves a method of suppressing the inhibition of neuron growth, comprising the steps of delivering to the nerve growth environment, antibodies directed against Rho family members in an amount effective to reverse said inhibition.

In accordance with another aspect of the present invention, there is provided an assay method useful to identify Rho family member antagonist agents that suppress inhibition of neuron growth, comprising the steps of:

5

a)   culturing neurons on a growth permissive substrate that incorporates a growth-inhibiting
        amount of a Rho family member; and

b)   exposing the cultured neurons of step a) to a candidate Rho family member antagonist agent
        in an amount and for a period sufficient prospectively to permit growth of the neurons;

thereby identifying as Rho family antagonists the candidates of step b) which elicit neurite outgrowth
from the cultured neurons of step a).

In accordance with another aspect of the present invention, there is provided a method to suppress
the inhibition of neuron, comprising the steps of delivering, to the nerve growth environment, a Rho
family antagonist in an amount effective to reverse said inhibition.

In another embodiment, the nucleic acids encoding Rho family members can be used in antisense
techniques and therapies.

In yet another embodiment, a kit is provided comprising components necessary to conduct the assay
method useful to screen Rho family antagonist agents.

Various other objects and advantages of the present invention will become apparent from the detailed
description of the invention.

BRIEF DESCRIPTION OF FIGURES

Figure 1: Treatment with C3 stimulates neurite outgrowth on inhibitory MAG substrates. A) PC12
cells plated on MAG remained rounded and did not extend neurites. B) Cells plated on MAG in the
presence of C3 grew neurites. C) PC12 cells plated on polylysine (PLL) substrates as a positive
control.

Figure 2. Role of integrins in overriding growth inhibition by myelin. The anti-α1 integrin function

blocking antibody, 3A3, was used to determine if integrin function is necessary for laminin to override growth inhibition by myelin or MAG. For experiments on myelin substrates (A-D), cells were fluorescently labelled with DiI, and plated on myelin (A), polylysine (B), or myelin +1 µg laminin (C and D). Control IgG was added to samples A-C, the 3A3 antibody to D. Neurites do not extend on myelin but grow on laminin or mixed laminin/myelin substrates. When 3A3 is added, laminin no longer overrides growth inhibition by myelin. Panels (E-H) show by phase contrast cells plated on recombinant MAG (E), laminin (F), or recombinant MAG plus laminin (G and H), with control antibody (E-G) or with 3A3 (H). Integrin function is needed to override growth inhibition by MAG.

Figure 3. PC12 cells transfected with dominant negative Rho extend short neurites on MAG substrates. Mock-transfected PC12 cells (a,c,e) or cells transfected with dominant-negative Rho (b,d,f) were plated on laminin (a,b) or MAG (c-f). MAG inhibits neurite outgrowth (c), but dominant negative Rho cells spread on MAG and some cells extend short neurites (d). Treatment with C3 further stimulates neurite outgrowth on MAG from both lines of cells (e,f).

Figure 4. Activation of Rho on MAG substrates. Activated Rho is associated with the plasma membrane. To determine if activated Rho was detected under conditions where PC12 cells do not grow neurites, cells were grown in suspension or plated on MAG or collagen substrates. Two hours later the plasma membranes were purified, the proteins separated by SDS PAGE, and the proteins transfered to nitrocellulose and stained with Ponceau S. Rho A was detected on the blots by immunoreactivity with anti-RhoA antibody. Immunoreactivity was strongest when cells were grown in suspension or when cells were plated on MAG. Therefore, Rho A is more active when cells are kept in suspension or plated on MAG than when plated on growth-permissive collagen.

DETAILED DESCRIPTION OF THE INVENTION

This invention arises from the discovery that Rho family members are key molecules in regulating inhibition by myelin proteins, and by MAG. Thus, this invention provides the advantage of identifying

7

an intracellular target, Rho family members, for all of the multiple inhibitory proteins that must be inactivated to allow for growth on myelin. The method of this invention provides for inactivation of Rho family members, thereby stimulating neurite growth on growth inhibitory substrates. Therefore, antagonists that inactivate Rho family members *in vivo* should allow axon regeneration in the injured or diseased CNS.

"Antagonist" refers to a pharmaceutical agent which in accordance with the present invention which inhibits at least on biological activity normally associate with Rho family members, that is blocking or suppressing the inhibition of neuron growth. Antagonists which may be used in accordance with the present invention include without limitation a Rho family members antibody or a binding fragment of said antibody, a Rho family members fragment, a derivative of Rho family members or of a Rho family members fragment, an analog of Rho family members or of a Rho family members fragment or of said derivative, and a pharmaceutical agent, and is further characterized by the property of suppressing Rho family members-mediated inhibition of neurite outgrowth.

The antagonist of Rho family members in accordance with the present invention is not limited to Rho family members or its derivatives, but also includes the therapeutic application of all agents, referred herein as pharmaceutical agents, which alter the biological activity of the Rho family members protein such that inhibition of neurons or their axon is suppressed.

The term "effective amount" or "growth-promoting amount" refers to the amount of pharmaceutical agent required to produce a desired antagonist effect of the Rho family members biological activity. The precise effective amount will vary with the nature of pharmaceutical agent used and may be *determined by one or ordinary skill in the art with only routine experimentation.*

As used herein, the Rho family of proteins comprises, but is not limited to rho, rac, cdc42 and their isotypes, such as RhoA, RhoB, and RhoC. Other members of the Rho family that are determined and whose inhibition of activity allows for neurite outgrowth are comtemplated to be part of this invention.

8

As used herein, the terms "Rho family member biological activity" refers to cellular events triggered by, being of either biochemical or biophysical nature. The following list is provided, without limitation, which discloses some of the known activities associated with contact-mediated growth inhibition of neurite outgrowth, adhesion to neuronal cells, and promotion of neurite out growth from new born dorsal root ganglion neurons.

As used herein, the term "biologically active", or reference to the biological activity of Rho family members or, or polypeptide fragment thereof, refers to a polypeptide that is able to produce one of the functional characteristics exhibited by Rho family members or its receptors described herein. In one embodiment, biologically active proteins are those that demonstrate inhibitory growth activities central nervous system neurons. Such activity may be assayed by any method known to those of skill in the art.

Based on the present evidence that Rho family members can affect growth inhibitory protein signals in myelin, the means exist to identify agents and therapies that suppress myelin-mediated inhibition of nerve growth. Further, one can exploit the growth inhibiting properties of Rho family members, or Rho family members agonists, to suppress undesired nerve growth. Without the critical finding that Rho family members has growth inhibitory properties, these strategies would not be developed.

Rho Family Member Antagonists and Assay Methods to Identify Rho family members Antagonists

In one embodiment, Rho family member antagonists will be inhibitors of GTPase activity. The *GTP/GDP cycle of Rho family members* activation/inactivation is regulated by a number of exchange factors. Compounds that block exchange, thereby inactivating Rho family members are preferred embodiments of this invention.

In another embodiment suitable Rho family member antagonist candidates are developed comprising fragments, analogs and derivatives of Rho family members. Such candidates may interfere with Rho

9

family members-mediated growth inhibition as competitive but non-functional mimics of endogenous Rho family members. From the amino acid sequence of Rho family members and from the cloned DNA coding for it, it will be appreciated that Rho family members fragments can be produced either by peptide synthesis or by recombinant DNA expression of either a truncated domain ofRho family members, or of intact Rho family members could be prepared using standard recombinant procedures, that can then be digested enzymically in either a random or a site-selective manner. Analogs of Rho family members or Rho family members fragments can be generated also by recombinant DNA techniques or by peptide synthesis, and will incorporate one or more, e.g. 1-5, L- or D-amino acid substitutions. Derivatives of Rho family members, Rho family members fragments and Rho family members analogs can be generated by chemical reaction of the parent substance to incorporate the desired derivatizing group, such as N-terminal, C-terminal and intra-residue modifying groups that have the effect of masking or stabilizing the substance or target amino acids within it.

In specific embodiments of the invention, candidate Rho family member antagonists include those that are derived from a determination of the functionally active region(s) of a Rho family member. The antibodies mentioned above and any others to be prepared against epitopes in Rho family members, when found to be function-blocking in *in vitro* assays, can be used to map the active regions of the polypeptide as has been reported for other proteins (for example, see Fahrig et al., (1993) *Europ., J. Neurosci.*, 5: 1118-1126; Tropak et al., (1994) *J. Neurochem.*, 62: 854-862). Thus, it can be determined which regions of Rho family members GTPases recognized by substrate molecules that are involved in inhibition of neurite outgrowth. When those are known, synthetic peptides can be prepared to be assayed as candidate antagonists of the Rho family members effect. Derivatives of these can be prepared, including those with selected amino acid substitutions to provide desirable properties to enhance their effectiveness as antagonists of the Rho family members candidate functional regions of Rho family members can also be determined by the preparation of altered forms of the Rho family members domains using recombinant DNA technologies to produce deletion or insertion mutants that can be expressed in various cell types as chimeric proteins that contain the Fc portion of immunoglobulin G (Kelm et al., (1994) *Curr. Biol.*, 4: 965-972). Alternatively, candidate mutant forms of Rho family members can be expressed on cell surfaces by transfection of various

10

cultured cell types. All of the above forms of Rho family members, and forms that may be generated by technologies not limited to the above, can be tested for the presence of functional regions that inhibit or suppress neurite outgrowth, and can be used to design and prepare peptides to serve as antagonists.

In accordance with an aspect of the invention, the Rho family member antagonist is formulated as a pharmaceutical composition which contains the Rho family member antagonist in an amount effective to suppress Rho family member-mediated inhibition of nerve growth, in combination with a suitable pharmaceutical carrier. Such compositions are useful, in accordance with another aspect of the invention, to suppress Rho family member-inhibited nerve growth in patients diagnosed with a variety of neurological disorder, conditions and ailments of the PNS and the CNS where treatment to increase neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, surgical nerve lesions or other CNS pathway lesions) damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, (1987) In: *Scientific American Medicines*, vol. 2, Scientific American Inc., N.Y., pp. 11-1-11-13) can be treated with such Rho family members antagonists. Examples of such disorders include but are not limited to Strokes, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler syndrome, scrapie, transmissible mink encephalopathy, Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, amylotrophic lateral sclerosis or Lou Gehrig's disease, progressive supranuclear palsy, Parkinson's disease and the like. The Rho family members antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of a Rho family member, or the binding portion thereof, or cells secreting such antibodies can also be used to inhibit Rho family member function in patients. In a particular embodiment of the invention, the Rho family members antagonist is used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

In another embodiment, the invention provides an assay method adapted to identify Rho family

member antagonists, that is agents that block or suppress the growth-inhibiting action of Rho family members. In its most convenient form, the assay is a tissue culture assay that measures neurite outgrowth as a convenient end-point, and accordingly uses nerve cells that extend neurites when grown on a permissive substrate. Nerve cells suitable in this regard include neuroblastoma cells of the NG108 lineage, such as NG108-15, as well as other neuronal cell lines such as PC12 cells (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA, ATCC accession NO. CRL 1721), human neuroblastoma cells, and primary cultures of CNS or PNS neurons taken from embryonic, postnatal or adult animals. The nerve cells, for instance about 10^3 cells-microwell or equivalent, are cultured on a growth permissive substrate, such as polylysine or laminin, that is over-layed with a growth-inhibiting amount of Rho family members. The Rho family members incorporated in the culture is suitably myelin-extracted Rho family members, although forms of Rho family members other than endogenous forms can be used provided they exhibit the Rhofamily members property of inhibiting neuron growth when added to a substrate that is otherwise growth permissive.

In this assay, candidate Rho family member antagonists, *i.e.*, compounds that block the growth-inhibiting effect of Rho family members, are added to the Rho family member-containing tissue culture preferably in amount sufficient to neutralize the Rho family member growth-inhibiting activity, that is between 1.5 and 15 μg of Rho family members antagonist per well containing a density of 1000 NG108-15 cells/well cultured for 24 hr. in Dulbecco's minimal essential medium. After culturing for a period sufficient for neurite outgrowth, e.g. 3-7 days, the culture is evaluated for neurite outgrowth, and antagonists are thereby revealed as those candidates which elicit neurite outgrowth. Desirably, candidates selected as Rho family member antagonists are those which elicit neurite outgrowth to a statistically significant extent, e.g., in at least 50%, more desirably at least 60%, e.g. 70%, per 1,000 cultured neurons.

Other assay tests that could be used include without limitation the following: 1) The growth cone collapse assay that is used to assess growth inhibitory activity of collapsin (Raper, J.A., and Kapfhammer, J.P., (1990) *Neuron*, 2:21-29; Luo et al., (1993) *Cell*, 75:217-227) and of various other

inhibitory molecules (Igarashi, M. et al., (1993) *Science*, 259:77-79) whereby the test substance is added to the culture medium and a loss of elaborate growth cone morphology is scored. 2) The use of patterned substrates to assess substrate preference (Walter, J. et al., (1987) *Development*, 101:909-913; Stahl et al., (1990) *Neuron*, 5:735-743) or avoidance of test substrates (Ethell, D.W. et al., (1993) *Dev. Brain Res.*, 72:1-8). 3) The expression of recombinant proteins on a heterologous cell surface, and the transfected cells are used in co-culture experiments. The ability of the neurons to extend neurites on the transfected cells is assessed (Mukhopadhyay et al., (1994) *Neuron*, 13:757-767). 4) The use of sections of tissue, such as sections of CNS white matter, to assess molecules that may modulate growth inhibition (Carbonetto et al., (1987) *J. Neuroscience*, 7:610-620; Savlo, T. and Schwab, M.E., (1989) *J. Neurosci.*, 9:1126-1133). 5) Neurite retraction assays whereby test substrates are applied to differentiated neural cells for their ability to induce or inhibit the retraction of previously extended neurites (Jalnink et al., (1994) *J. Cell Bio.*, 126:801-810; Sudan, H.S. et al., (1992) *Neuron*, 8:363-375; Smalheiser, N. (1993) *J. Neurochem.*, 61:340-342). 6) The repulsion of cell-cell interactions by cell aggregation assays (Kelm, S. et al., (1994) *Current Biology*, 4:965-972; Brady-Kainay, S. et al., (1993) *J. Cell Biol.*, 4:961-972). 7) The use of nitrocellulose to prepare substrates for growth assays to assess the ability of neural cells to extend neurites on the test substrate (Laganeur, C. and Lemmon, V., (1987) *PNAS*, 84:7753-7757; Dou, C-L and Levine, J.M., (1994) *J. Neuroscience*, 14:7616-7628).

Useful Rho family member antagonists include antibodies to Rho family members and the binding fragments of those antibodies. Antibodies which are either monoclonal or polyclonal can be produced which recognize Rho family members and its various epitopes using now routine procedures. For the raising of antibody, various host animals can be immunized by injection with Rho family members or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinmitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin).

13

Production of Antibodies Against the Components of Rho Family Members

Antibodies can be produced which recognize members of the Rho family. Such antibodies can be polyclonal or monoclonal. Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of Rho family members. For the production of antibody, various host animals can be immunized by injection with a neurite growth regulatory factor protein, or a synthetic protein, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. A monoclonal antibody to an epitope of a Rho family member can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In a particular embodiment, the procedure described . may be used to obtain mouse monoclonal antibodies which recognize Rho family members.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (.RTM..q., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452). A molecular clone of an antibody to a neurite growth regulatory factor epitope can be prepared by known techniques. Recombinant DNA

14

methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) may be used to construct nucleic acid sequences which encode a monoclonal antibody olecule, or antigen binding region thereof.

A monoclonal antibody to an epitope of a Rho family member can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Köler and Milstein ((1975) *Nature*, 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., (1983) *Immunology Today*, 4:72) and EBV-hybridoma technique (Cole et al., (1985) In *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp 77-96). In a particular embodiment, the procedure described by Nobile-Orazio et al. ((1984) *Neurology*, 34:1336-1342) may be used to obtain antibodies which recognize recombinant Rho family members (for example of techniques, see Attia S. et al., (1993) *J. Neurochem.*, 61: 718-726).

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g. Tan et al., (1983) *Proc. Natl. Acad. Sci. U.S.A.*, 80: 7308-7312; Kozbor et al., (1983) *Immunology Today, 4: 72-79; Olsson et al.*, (1982) *Meth. Enzymol.*, 92: 3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human contact regions (Morrision et al., (1984) *Proc. Natl. Acad. Sci. U.S.A.*, 81: 6851; Takeda et al., (1985) *Nature*, 314: 452).

A molecular clone of an antibody to a Rho family member epitope can be prepared by known techniques. Recombinant DNA methodology may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof (see e.g., Maniatis et al., (1982) In *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

For use, Rho family member antibody molecules may be purified by known techniques, such as

15

immunoabsorption or immunoaffinity chromatography, chromotographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Rho family member antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F (ab')$_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab, fragments which cen be generated by reducing the disulfide bridges of the F (ab')$_2$ fragment, and the two Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Monoclonal antibodies known to react with human Rho family members may be tested for their usefulness to serve as Rho family member antagonists (Nobile-Orazio et al., (1984) *Neurology*, 34: 1336-1342; Doberson et al., (1985) *Neurochem. Res.*, 10: 499-513).

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab').sub.2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab, fragments which can be generated by reducing the disulfide bridges of the F(ab').sub.2 fragment, and the 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Diagnostic, Therapeutic and Research Uses for Rho Family Member Antagonists and Antibodies

Rho family member antagonists and antibodies have uses in diagnostics. Such molecules can be used in assays such as immunoassays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth extension, invasiveness, and regeneration. Alternatively, the Rho family member antagonists and antibodies may be used to monitor therapies

16

for diseases and conditions which ultimately result in nerve damage; such diseases and conditions include but are not limited to CNS trauma, (e.g. spinal cord injuries), infarction, infection, malignancy, exposure to toxic agents, nutritional deficiency, paraneoplastic syndromes, and degenerative nerve diseases (including but not limited to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, amyotrophic lateral sclerosis, progressive supra-nuclear palsy, and other dementias). In a specific embodiment, such molecules may be used to detect an increase in neurite outgrowth as an indicator of CNS fiber regeneration. For example, in specific embodiments, altered levels of Rho family members in a patient sample containing CNS myelin can be a diagnostic marker for the presence of a malignancy, including but not limited to glioblastoma, neuroblastoma, and melanoma, or a condition involving nerve growth, invasiveness, or regeneration in a patient. In a particular embodiment, altered levels of Rho family members can be detected by means of an immunoassay in which the lack of any binding to anti-inhibitory protein antibodies is observed. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, precipitation reactions, gel diffusion precipitation reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, immunoelectrophoresis assays, and immunohistochemistry on tissue sections, to name but a few.

Useful for nerve growth suppression are pharmaceutical compositions that contain, in an amount effective to suppress nerve growth, either Rho family member antagonist or antibody in combination with an acceptable carrier. Candidate Rho family members antagonists include fragments of Rho family members that incorporate the ectodomain, including the ectodomain *per se* and other N- and/or C-terminally truncated fragments of Rho family members or the ectodomain, as well as analogs thereof in which amino acids, e.g. from 1 to 10 residues, are substituted, particularly conservatively, and derivatives of Rho family members or Rho family members fragments in which the N- and/or C-terminal residues are derivatized by chemical stabilizing groups. Such Rho family members antagonists can also include anti-idiotypes of Rho family members antibodies and their binding

17

fragments.

In specific embodiments of the invention, candidate Rho family members antagonists include specific regions of the Rho family members molecule, and analogs or derivatives of these. These can be identified by using the same technologies described above for identification of Rho family members regions that serve as inhibitors of neurite outgrowth.

The Rho family members related derivatives, analogs, and fragments of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, Rho family members-encoding DNA can be modified by any of numerous strategies known in the art (Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), such as by cleavage at appropriate sites with restriction endonuclease(s), subjected to enzymatic modifications if desired, isolated, and ligated *in-vitro*.

Additionally, the Rho family members-encoding gene can be mutated *in-vitro* or *in-vivo* for instance in the manner applied for production of the ectodomain, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in-vitro* modification. Any technique for mutagenesis known in the art cab be used, including but not limited to, *in-vitro* site directed mutagenesis (Hutchinson, *et al.*, J. Biol. Chem., 253, 6551, 1978), use of TAB™ linkers (Pharmacia), etc.

For delivery of Rho family members antagonists, various known delivery systems can be used, such as encapsulation in liposomes or semipermeable membranes, expression in suitably transformed or transfection glial cells, oligodendroglial cells, fibroblasts, etc. according to the procedure known to those skilled in the are (Lindvall *et al.*, Curr. Opinion Neurobiol., 4, 752-757, 1994). Linkage to ligands such as antibodies can be used to target delivery to myelin and to other therapeutically relevant sites *in-vivo*. Methods of introduction include, but are not limited to, intradermal,

18

intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and transfusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting Rho family members antagonist activity, for example, and not by way of limitation, hybridoma cells encapsulated in a suitable biological membrane may be implanted in a patient so as to provide a continuous source of Rho family members inhibitor.

Therapeutic Uses of Rho family members

CNS myelin associated inhibitory proteins of the present invention can be therapeutically useful in the treatment of patients with malignant tumors including, but not limited to melanoma or tumors of nerve tissue (e.g. neuroblastoma). In one embodiment, patients with neuroblastoma can be treated with Rho family members or analogs, derivatives, or subsequences thereof, and the human functional equivalents thereof, which are inhibitors of neurite extension.

In an alternative embodiment, antagonists, derivatives, analogs, inhibitors, or antibodies to Rho family members can be used in regimens where an increase in neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, or other CNS pathway lesions), surgical nerve lesions, damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, 1987, In: Scientific American Medicines v. 2, Scientific American Inc., N.Y., pp. 11-1-11-13) can be treated with such inhibitory protein antagonists. Examples of such disorders include but are not limited to Alzheimer's Disease, Parkinsons' Disease, Huntington's Chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy and other dementias. Such antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of, (or the binding portion thereof, or cells secreting such as antibodies) can also be used to inhibit Rho family members protein function in patients. In a particular embodiment of the invention, antibodies directed to Rho family members may be used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

19

Various delivery systems are known and can be used for delivery of antagonists or inhibitors of Rho family members, related molecules, or antibodies thereto, e.g., encapsulation in liposomes or semipermeable membranes, expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target myelin associated protein-related molecules to therapeutically desirable sites in vivo. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and infusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting CNS myelin inhibitory protein antagonist activity, for example, and not by way of limitation, hybridoma cells, encapsulated in a suitable biological membrane may be implanted in a patient so as to provide a continuous source of anti-CNS myelin inhibiting protein antibodies.

In addition, any method which results in decreased synthesis of Rho family members may be used to diminish their biological function. For example, and not by way of limitation, agents toxic to the cells which synthesize Rho family members and/or its receptors (e.g. oligodendrocytes) may be used to decrease the concentration of inhibitory proteins to promote regeneration of neurons.

EXAMPLES

EXAMPLE 1

This example demonstrates *in vitro* evidence that Rho family members are responsible for regulating the neuronal response to MAG. In particular, this demonstrates that the small GTPase Rho regulates the response to MAG. PC12 cells were plated on polylysine (PLL), laminin, or MAG substrates and *exposed to NGF to stimulate* neurite growth. PC12 cells differentiated neurites on PLL and laminin substrates, but on MAG substrates the cells remained rounded and did not grow neurites.
The addition of the ADP-ribosyl transferase C3 from *Clostridium botulinum*, that efficiently inactivates Rho family members without affecting Rac and cdc42 (Udagawa and McIntyre, 1996), allowed the cells to extend neurites on MAG substrates. In addition this example demonstrates neurite growth from PC12 cells transfected with a dominant negative N19RhoA construct. On laminin

and PLL substrates the N19 RhoA PC12 cells grew neurites that were longer than the mock-transfected controls. Moreover, N19 RhoA PC12 cells were able to extend neurites when plated on MAG substrates. Therefore, the inactivation of Rho stimulates neurite outgrowth and allows neurite extension on MAG substrates. These results implicate Rho in signaling growth inhibition by MAG

Cell Culture

We obtained PC12 cells from three different sources: from Dr. Phil Barker (Montreal Neurological Institute); from the ATCC (obtained from W. Mushinsky, McGill), and from Gabor Tigyi, (University of Tennessee) and we found that all lines of cells were inhibited by both myelin and MAG. PC12 cells were grown in Dulbecco's modified eagle's medium (DMEM) with 10 % horse serum and 5 % fetal bovine serum. PC12 cells stably transfected with constitutively active and dominant negative RhoA constructs were kindly provided by Dr. G. Tigyi (University of Tennessee, Memphis, USA). The three cell lines used included a mock transfected cell line, a constitutively active RhoA (V14GRhoA) cell line, and a dominant negative RhoA (N19TRhoA) cell line. Transfected PC12 cell lines were maintained in the growth medium containing 400 mg/L G418. For cell differentiation experiments, cells were plated on appropriate substrates in DMEM with 1 % fetal bovine serum and 100 ng/ml nerve growth factor. For experiments on mixed substrata (laminin/MAG or laminin/myelin), PC12 were plated in DMEM with 1% lipid free-BSA in the presence or the absence of 50µg/ml of an irrelevant antibody or of a purified function blocking antibody (clone 3A3) against the rat $\alpha 1\beta 1$ integrin (a gift of S.Carbonetto). PC12 cell differentiation experiments were done in 96-well plates in duplicate, and each experiment was repeated a minimum of three times.

To culture cerebellar granule cells, 3 - 4 rats from P3 to P7 were decapitated. The cerebellum was removed and placed in MEM-HEPES where underlying tissue and the meninges was removed. The cerebellum was cut into small pieces and treated with 0.125% trypsin in MEM-HEPES for 20' at 37°C. The tissue was then triturated with a fire polished pasteur pipette to break up any clumps of tissue. The cells were spun down at 1500 rpm for 10', and the pellet was resuspended in MEM-HEPES with 2mM EDTA. The cell suspension was placed on an iso-osmotic percoll gradient with

21

60% and 35% percoll, centrifuged for 15' at 2300 rpm, and the interface between the 60% and 35% percoll was collected. Cells were washed once, and resuspended in DMEM with 10% FBS, vitamins, and penicillin/streptomycin in the presence or absence of 20 μg/ml C3 transferase. Cells were placed in 4-chamber, chamber slides coated with poly-l-lysine or laminin and treated with spots of MAG or myelin. 200,000 cells per chamber were plated.

Preparation of growth substrates

Poly-l-lysine was obtained from Sigma (St. Louis, Mo). Laminin was prepared from EHS tumors (Paulsson and Lindblom (1994). Cell biology: A laboratory handbook, Academic Press, pp589-594) and collagen from rat tails (Greene et al (1987) Meth. Enzymology 147:207-216). Myelin was made from bovine brain corpus callosum, and native MAG was purified from myelin after extraction in 1% octylglucoside and separation by ion exchange chromatography (McKerracher et al (1994) Neuron 13:801). This native MAG has some additional proteins, including some tenascin (Xiao et al !997) Neurosci. Abstr vol 23:1994). Recombinant MAG was made in baculovirus as described (McKerracher et al, IBID).

Test substrate were prepared as uniform substrates in 96-well plates or 4-chambered slides, or as spots on 18 mm glass coverslips. First, poly-L-lysine was coated by incubation of 100 μg/ml for 3 hours at 37°C, and the wells or coverslips were washed with water and dried. Laminin substrates were prepared by incubating 25 μg/ml laminin on poly-L-lysine coated dishes for 3 hours at 37°C. Solid MAG or myelin substrates were prepared by drying down MAG overnight, or incubating a 10 mg/ml myelin solution for 3 hours on polylysine coated substrates. For 96-well plates, 1-4 μg of either recombinant MAG (rMAG) or of native MAG per well was used. For mixed laminin/myelin or laminin/MAGsubstrata, 8μg of inhibitory proteins and 10 μg of laminin were dried down on 96-well plates precoated with polylysine. For 4-chambered chamber slides, 40 μg MAG per chamber was used, and for 100 mm plates 0.6-1 mg of MAG was dried down. Spots of MAG on coverslips were generated by plating of 2 mg/ml recombinant MAG on polylysine for 3-4 hours in a humid chamber at 37°C. Collagen substrates were made by incubating 10-15 μg/ml of rat tail collagen for

22

3 hours at 37°C.

Immunocytochemistry

PC12 cells were visualized by phase contrast microscopy, or following labelling with the lipophilic fluorescent dye, DiI (McKerracher et al, 1994). Granule cells were visualized by immunocytochemistry. Following 12-24 hours in culture, cells were fixed for 30' at room temperature in 4% paraformaldehyde, 0.5% glutaraldehyde, 0.1 M phosphate buffer. Following fixation, cells were washed 3 X 5' with PBS and then blocked for 1 hour at room temperature in 3%BSA, 0.1% Triton-X 100. Granule cell cultures were incubated overnight with a polyclonal anti-rMAG antibody (called 57A++) to label MAG spots. The MAG antibody was detected using an FITC conjugated secondary antibody. Rhodamine conjugated phalloidin was diluted 1:200 with the secondary antibody to label granule cell actin filaments.

C3 transferase preparation and use

The plasmid pGEX2T-C3 coding for the GST-C3 fusion protein was obtained from A. Hall (London). Recombinant C3 was purified as described by Dillon and Feig (Met. Enzymology, (1994), 256, pp 174-184). After fusion protein cleavage by thrombin, thrombin was removed by incubating the protein solution 1 hour on ice with 100μl of p-aminobenzamidine agarose-beads (Sigma). The C3 solution was desalted on PD10 column (Pharmacia) with PBS, and sterilized through a 0.22μm filter. The C3 concentration was evaluated by Lowry assay (DC protein assay, Bio-Rad) and toxin purity was controlled by SDS-PAGE analysis.

To test the effect of C3 on the outgrowth on PC12 cells, C3 transferase was scrape loaded into the cells before plating on appropriate substrates. Cells were grown to confluence in serum containing media in 6 well plates. Cells were washed once with scraping buffer (114mM KCl, 15mM NaCl, 5.5 mM MgCl$_2$, 10mM Tris-HCl). Cells were then scraped with a rubber policeman into 0.5 ml scraping buffer in the presence or absence of 20 μg/ml C3 transferase. The cells were pelleted, and

resuspended in 2 ml DMEM, 1% FBS, and 50 ng/ml nerve growth factor before plating. 10 μg/ml C3 was added to scrape loaded cells. Cells were differentiated for 48 hours then fixed in 4 % paraformaldehyde, 0.5 % glutaraldehyde, 0.1 M PO_4 buffer.

Membrane Translocation Assay for RhoA

PC12 cells were collected and resuspended in DMEM, 0.1 % BSA, 50ng/ml NGF, then plated on 100 mm dishes coated with collagen or MAG, or left in suspension. Two hours later, cells were washed with ice cold PBS + protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin). Cells were then scraped into 5ml PBS + protease inhibitors, and the cells were pelleted and washed with PBS + protease inhibitors. The cell pellets were mechanically homogenized by 25 strokes in a glass-teflon homogenizer, the homogenate centrifuged for 20 min at 8,000 rpm, and the cell debris in the pellet was discarded. The supernatant was centrifuged for 1 hour at 100,000 x g to separate membrane and cytosolic fractions. Membrane pellets were washed 1 X with PBS + protease inhibitors and resuspended in PBS with 0.5 % SDS, and 50-100 μg of membrane protein was analyzed by SDS-PAGE on 12 % gels. Gels were transferred to Protran nitrocellulose membrane and stained with Ponceau S. Blots were blocked for 1 hour in 5 % skim milk in TBS, and probed overnight with Rho A antibody diluted 1:200 in 1.5 % skim milk in TBS. Rho A antibody was detected by using an alkaline phosphatase conjugated secondary antibody and an alkaline phosphatase detection kit (Gibco-BRL).

Growth inhibition of PC12 cells and its modulation by NGF and laminin

PC12 cells typically extend neurites in response to NGF, but when plated on myelin substrates the cells remain round and do not extend neurites (Moskowitz et al. 1997) (Fig. 2). MAG is a potent inhibitor of axon growth present in myelin. We observed that PC12 cells plated on substrates of MAG also remained rounded (Fig.1), a finding in contrast to a report that PC12 cells are not responsive to MAG (Bartsch et al. 1995). To further examine the response of PC12 cells to MAG, we plated three different lines of PC12 cells on both native and recombinant MAG substrates in the presence of NGF.

24

All of the lines of PC12 cells showed reduced cell spreading, and most cells remained rounded without neurites. However, with increasing time, some neurites were able to extend on MAG substrates (see below). We also observed that different preparations of MAG can differ in their potency to inhibit neurite growth, and that the activity of MAG is reduced or lost upon freeze-thaw.

Laminin is known to override completely, growth inhibition of NG108 cells by myelin (David et al. 1995). Similarly, we found that PC12 cells are able to extend neurites on mixed myelin and laminin substrates or on mixed laminin/MAG substrates (Fig.2). To determine if signalling through integrins is responsible for overriding growth inhibition by myelin, we used the integrin function blocking antibody 3A3 raised against the $\alpha 1$ subunit extracellular domain. Previous studies have documented that $\alpha 1 \beta 1$ integrin is the dominant integrin expressed by PC12 cells, and that the 3A3 antibody blocks PC12 cell neurite growth on laminin (Tomaselli et al. 1990). We plated PC12 cells on mixed myelin and laminin substrates, in the presence of the 3A3 antibody, or with a non-specific IgG antibody as a control. The 3A3 antibody blocked neurite extension on both and laminin and the mixed myelin/laminin substrates (Fig. 2). On MAG or on myelin substrates the cells remained rounded. The observation that the 3A3 antibody restores growth inhibition on mixed substrates demonstrates that laminin does not override growth inhibition by masking the inhibitory domain of MAG, but that signals elicited through integrins receptors are responsible.

Effect of C3 Transferase on PC12 cells

To investigate possible intracellular targets that may override growth inhibition by myelin and by MAG, we focused on the small GTPase Rho which is known to play a role in convergent signalling *pathways that affect* morphology and motility (Hall, 1996). We inactivated Rho in PC12 cells by scrape loading them with the bacterial toxin C3 before plating the cells on the test substrates. C3 is known to inactivate Rho through ADP ribosylation (Udagawa and McIntyre, 1996). On control substrates of polylysine and laminin, treatment with C3 potentiated both the number of cells with neurites and the length of neurites from cells (Fig.3). On MAG and myelin substrates where neurite formation is inhibited, C3 has a dramatic effect on the ability to extend neurites (Fig 3). When treated

with C3, about half of the PC12 cells plated on either rMAG or native MAG had neurites of approximately 1 cell body diameter. In contrast, the untreated cells remained rounded and clumped. Similarly, PC12 cells plated on myelin remained rounded, but the addition of C3 allowed neurites to extend directly on the myelin substrate. These results demonstrate that C3 treatment elicits neurite growth from PC12 cells plated on growth inhibitory myelin or MAG substrates.

Growth of dominant-negative Rho-transfected cells on MAG substrates

PC12 cells transfected with constitutively active RhoA (V14GRhoA), and PC12 cells transfected with dominant negative RhoA (N19TRhoA), and the mock-transfected cells, were examined for their ability to extend neurites on different test substrates. Cells with the constitutively active mutation, V14GRhoA cells, differentiated poorly on all substrates, including poly-L-lysine and laminin. The treatment of the V14GRhoA cells with C3 allowed the growth of some short neurites on all of the test substrates, including MAG.

In the same series of experiments the response of dominant negative Rho- transfected cells, N19TRhoA cells, to MAG and myelin substrates was examined. When N19TRhoA cells were plated on MAG substrates, they spread and did not remain rounded as did the mock transfected PC12 cells. A small number of cells had short neurites, an effect that was observed on both the rMAG and native MAG substrates (Fig.3).

C3 treatment of mock transfected and N19TRhoA cells had a dramatic effect of neurite outgrowth as most cells had extensive neurites (Fig.3). The effect of C3 on N19TRhoA cells was much more *marked than the effect* on the mock transfected cells. Therefore, the combination of C3 treatment and transfection of dominant negative Rho elicited excellent outgrowth of neurites from PC12 cells plated on inhibitory MAG (and myelin) substrates.

Effect of C3 on Primary Cells

26

To test the involvement of Rho in the response of primary neurons to MAG and to myelin substrates, cerebellar granule neurons were plated on test substrates and treated with C3. Neurite outgrowth from these cells was known to be inhibited by MAG (Li et al. 1996) and the C3 stimulated growth of neurites from the granule cells on both permissive and inhibitory substrates.

The growth substrate influences the cellular location of Rho

Rho is associated with the plasma membrane when it is in an activated state, and it moves into the cytosolic fraction when it is in the GDP-bound inactive state. To determine if the growth substrate influences the cellular localization of Rho, cells were either left in suspension or plated on MAG or collagen substrates, and prepared membranes from the cells two hours later. It was shown that Rho was principally localized in the cytosolic fraction when cells were plated on collagen, a growth permissive substrate. However, Rho was associated with the plasma membrane when cells where grown in suspension and when cells were plated on MAG (Fig. 4).

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28

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30

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications to the invention to adapt it to various usages and conditions. Such changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims.
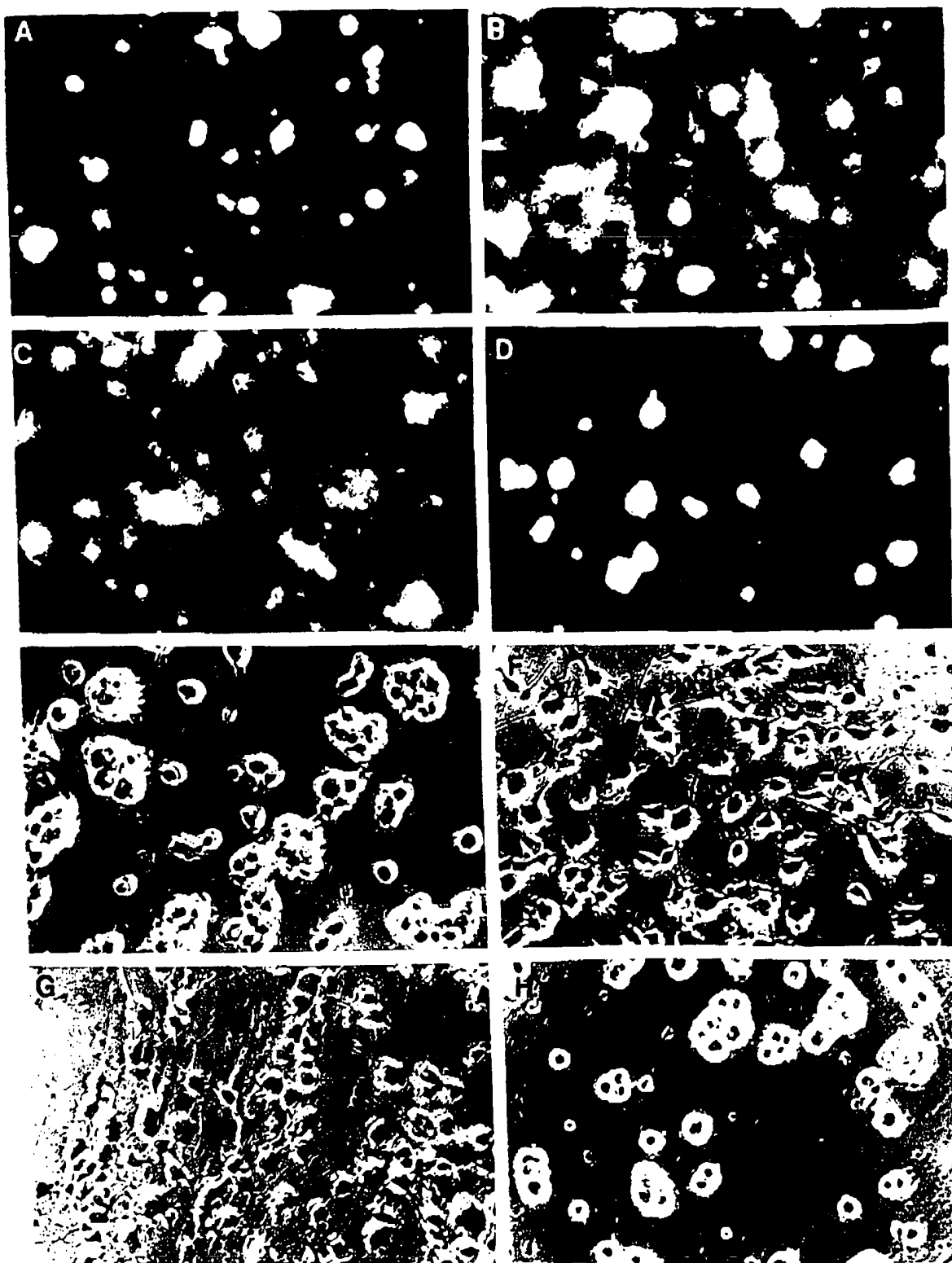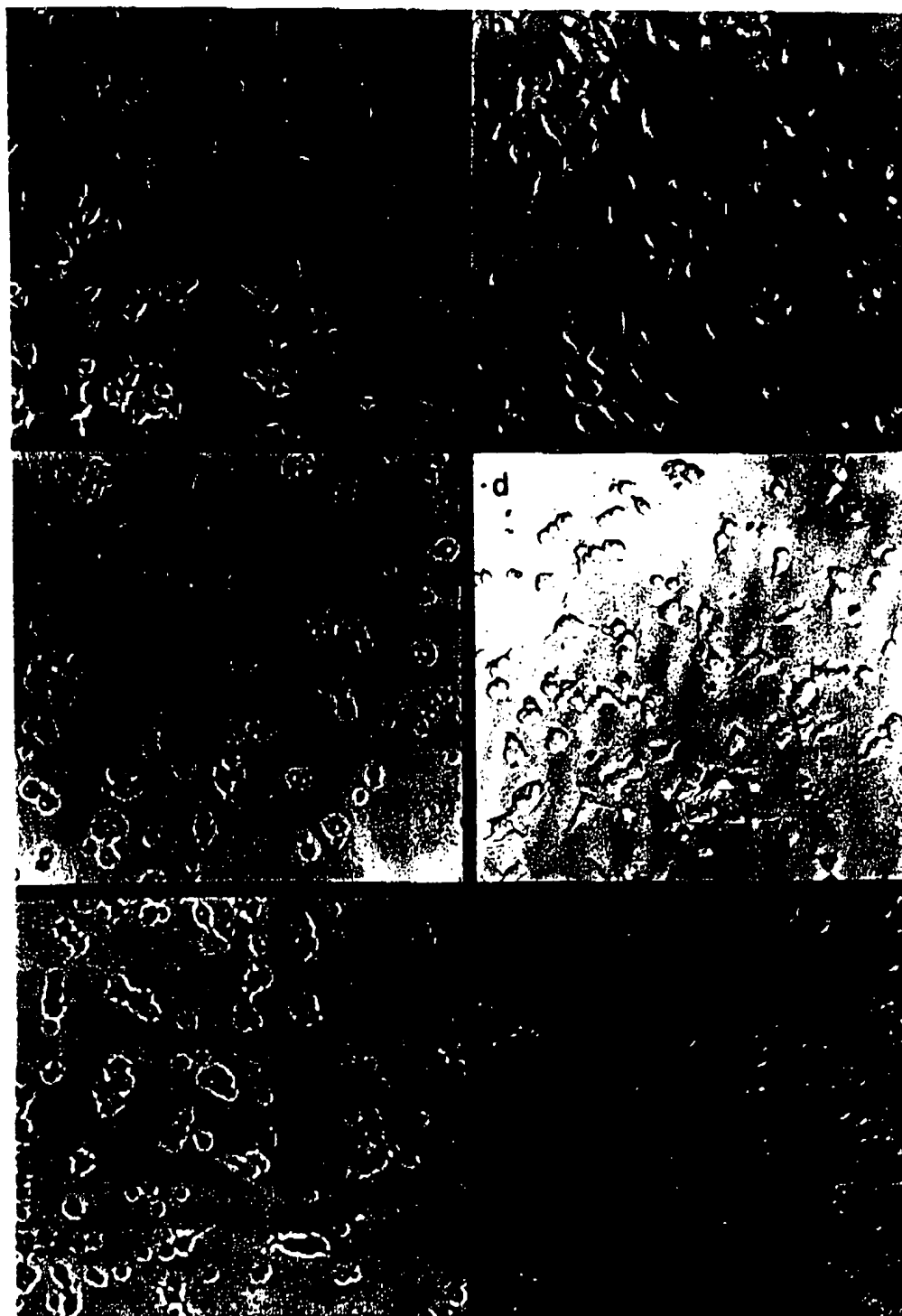
MAG

MAG
+
C3

PLL
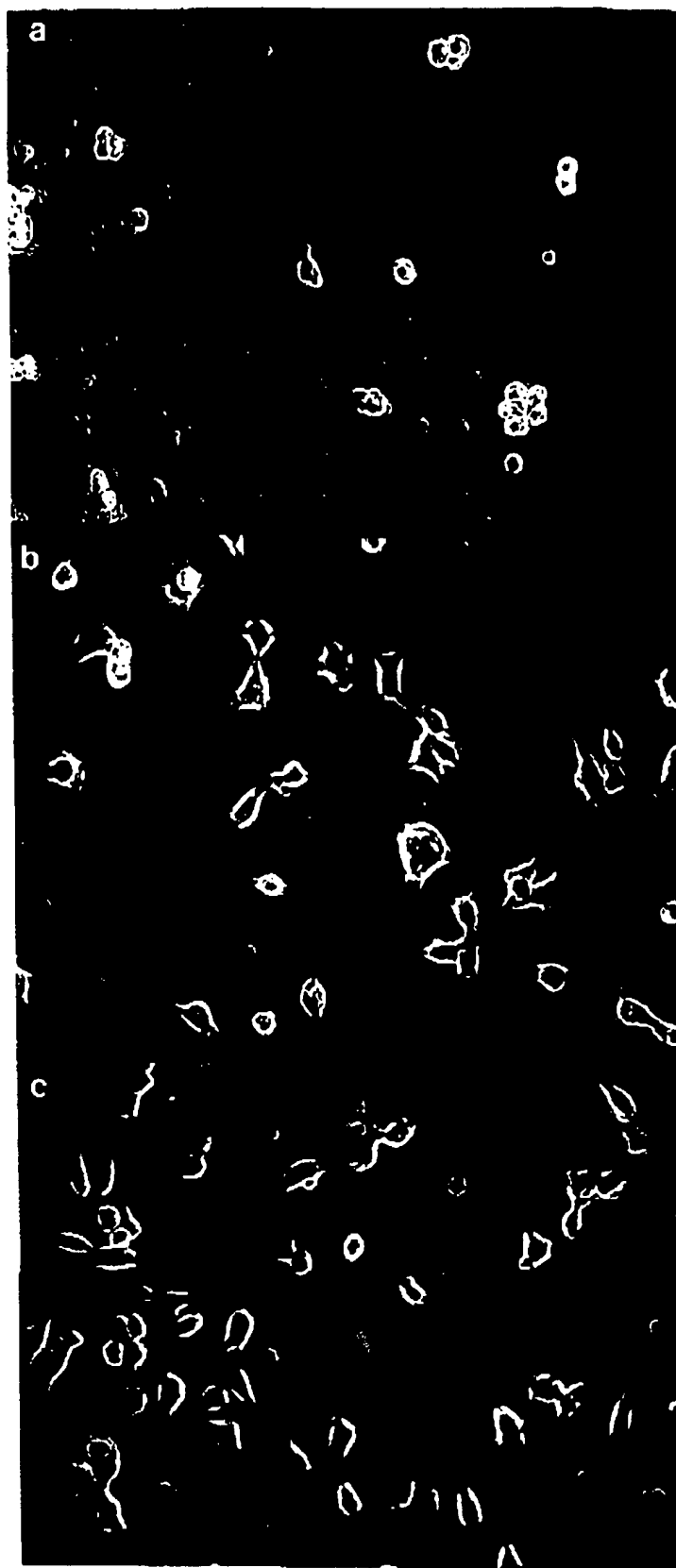
Figure 1

Figure 2

Mock PC12   Dominant Negative PC12
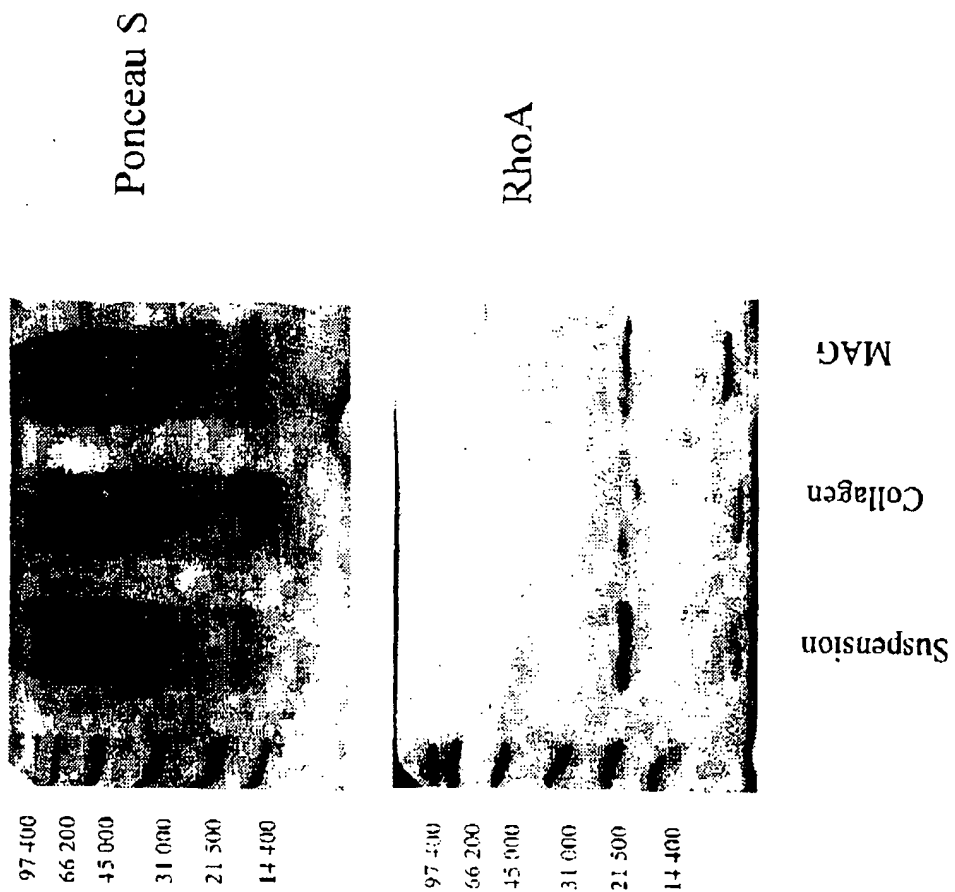
Laminin

MAG

Figure 3

MAG + C3

Mock PC12   Dominant Negative PC12

Figure 4